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Epidemiology of Virulence-Associated Plasmids and Outer Membrane Protein Patterns Within Seven Common *Salmonella* Serotypes

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Antibiotic-sensitive *Salmonella* isolates belonging to seven common serotypes and originating from 29 different countries from all continents were investigated for their plasmid DNA content (337 isolates) and their outer membrane protein profiles (216 isolates). Of the *S. typhimurium*, *S. enteritidis*, *S. dublin*, and *S. choleraesuis* isolates, 90% or more carried a serotype-specific plasmid. The molecular sizes of the plasmids were 60 megadaltons (Md) for *S. typhimurium*, 37 Md for *S. enteritidis*, 56 Md for *S. dublin*, and 30 Md for *S. choleraesuis*. The outer membrane protein profiles were homogeneous within each of the seven serotypes, except that a minority of *S. enteritidis* and *S. dublin* strains were lacking one major outer membrane protein. Virulence studies were performed with 39 representative strains by measuring the 50% lethal doses (LD_{50}s) after oral infection of mice. The LD_{50} values obtained for plasmid-positive strains of *S. typhimurium*, *S. enteritidis*, and *S. dublin* were up to 10^6-fold lower than the values obtained for the plasmid-free strains of the same serotype. Only the plasmid-positive strains could invade the livers of orally infected mice, and only they were resistant to the bactericidal activity of 90% guinea pig serum. Strains of *S. infantis* were generally plasmid free, whereas *S. panama* and *S. heidelberg* isolates carried heterogeneous plasmid populations. The virulence properties of the latter three serotypes could not be correlated with the predominant plasmids found in these strains.

Bacterial plasmids contribute a wide variety of phenotypes to their bacterial host, including antibiotic resistance and virulence properties (for a review, see reference 4). Many analyses of *Salmonella* plasmids have concentrated on the epidemiology and molecular properties of plasmids originating from antibiotic-resistant strains. These analyses have revealed that in a given geographic area an individual bacterial clone predominates (11, 30). Until now there has been no world-wide epidemiological analysis of plasmids carried by antibiotic-sensitive *Salmonella* strains. Because similar studies performed on other enteric bacteria established that plasmids can be responsible for pathogenesis, (20, 24, 25), it seemed that the plasmid DNA analysis of a large number of strains could lead to the detection of candidates for *Salmonella* virulence plasmids. Their existence has already been indicated for *S. typhimurium* (12) and *S. dublin* (29).

Analyses of multiresistant *S. dublin* isolates (26) and of *S. choleraesuis* bacteria from Germany indicated that within these serotypes all isolates tested carried a plasmid of the same molecular weight. This led us to investigate the plasmid DNA content of antibiotic-sensitive *Salmonella* isolates of seven common serotypes from all over the world. In addition to these studies, previous epidemiological studies of the major outer membrane protein profiles of *Escherichia coli* (1), *Haemophilus influenzae* (2), and *Neisseria* species (5, 18) proved to be very useful markers for the investigation of clonal descent. Outer membrane proteins can, like plasmids, influence the virulence and immunological properties of bacteria (5, 10, 31). Consequently, we included a detailed analysis of the outer membrane proteins of *Salmonella* species in our study as well.

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The aim of the study was to establish the world-wide epidemiological relationships within seven important serotypes and to investigate the virulence properties of the predominating bacterial clones.

Our study focused on *S. typhimurium*, *S. dublin*, *S. enteritidis*, *S. infantis*, *S. heidelberg*, *S. panama*, and *S. choleraesuis*. These serotypes have been shown to be the major causes of salmonellosis in humans and animals throughout the world (23; E. Kelterborn, VII Int. Colloq. Lab. Methods Epidemiol. Surveill., 1981, abstr.). *S. typhimurium*, *S. enteritidis*, *S. dublin*, and *S. choleraesuis* were found to represent one bacterial clone which was characterized by the possession of a virulence plasmid and a homogeneous outer membrane protein profile. We also show that in these serotypes, avirulent subclones existed which were derived from the major clones by the loss of their respective virulence-associated plasmids.

MATERIALS AND METHODS

Media. L-broth and L-agar plates (17) served as growth media. Gassner plates were prepared by the method of Gassner (9).

Bacterial strains. Strains were received from 33 different laboratories, as stab cultures or as filter papers soaked in glycerol cultures. After receipt, they were immediately inoculated into 5 ml of L-broth and incubated at 37°C overnight. Subsequently, glycerol cultures were prepared by the addition of 5 ml of 80% (vol/vol) glycerol (Merck, Darmstadt, Federal Republic of Germany [FRG]) in L-broth and were stored at −30°C.

All strains were tested for their susceptibility to ampicillin, tetracycline, chloramphenicol, kanamycin, and trimethoprim by agar diffusion tests with antibiotic disks (Oxoid Ltd., London, England). Resistant strains were excluded from the study. The antibiotic-susceptibility test also al-

lowed the detection of contaminated strains, which were repurified. Such repurified isolates, all isolates which were plasmid free or carried plasmids other than the serotype-specific plasmids described later, were serotyped by slide agglutination. Diagnostic O and H antisera prepared as described previously (8) were used for this purpose.

Spontaneous nalidixic acid-resistant (Nal^r) derivatives of strains were selected on L-agar plates containing 25 μg of nalidixic acid (Serva, Heidelberg, FRG) per ml. All resistant clones used for further experiments were tested for their serotype, plasmid content, and virulence properties in mice. No difference in these properties between an Nal^r derivative and its parent strain was ever detected.

Strains were received from the following individuals: S. Dixon, Salmonella Reference Laboratory, Adelaide, Australia; J. Bensink, University of Queensland, Brisbane, Australia; G. Ghysels, Institut voor Hygiene en Epidemiology, Brussels, Belgium; E. Hofer, Ministerio da Saude, Rio de Janeiro, Brazil; G. Pessôa, Instituto Adolfo Lutz, Sao Paulo, Brazil; H. Lior, Laboratory Centre for Disease Control, Ottawa, Canada; O. Pietzschi, Bundesgesundheitsamt, Berlin, FRG; J. Gledel, Ministère de l'Agriculture, Paris, France; M. Jahkola, National Public Health Institute, Helsinki, Finland; C. Wray, Central Veterinary Laboratory, Weybridge, United Kingdom; S. Saxena, Central Research Institute, Kasauli, India; M. Mazzotti, Instituto Superiore di Sanita, Rome, Italy; G. Allos, Ministry of Health, Baghdad, Iraq; J. Sechter, Vaccine and Serum Institute, Jerusalem, Israel; A. Kohn, Laboratoire de Médecine Vétérinaire, Luxembourg, Luxembourg; M. Jegathesan, Institute for Medical Research, Kuala Lumpur, Malaysia; G. Purev, National Reference Laboratory on Salmonella, Ulan Bator, Mongolian People's Republic; J. Lassen, National Institute of Public Health, Oslo, Norway; N. van Leeuwen, Rijks Instituut voor de Volksgezondheid, Bilthoven, Netherlands; M. Kourany, Grogas Memorial Laboratorio, Panama City, Panama; O. Grados, Instituto de Salud Pública, Lima, Peru; O. Bazalar, Instituto Nationales de Salud, Lima, Peru; R. Glosnicka, Institute of Maritime and Tropical Medicine, Gdynia, Poland; M. Negut, Institutul Cantacuzino, Bucharest, Romania; Y. Buisson, Institut Pasteur, Dakar, Senegal; J. Merino, Centro National de Salmonellosis, Madrid, Spain; A. Lindberg, National Bacteriological Laboratory, Stockholm, Sweden; B. Hurvell, National Veterinary Institute, Uppsala, Sweden; H. Fey, Universität Bern, Bern, Switzerland; P. Ekachampaka, National Salmonella Centre, Bangkok, Thailand; D. Brenner and A. Murlin, Centers for Disease Control, Atlanta, Georgia; J. Toure, Centre Muraz, Bobo-Dioulasso, Upper Volta; and N. Stosić, Institute of Public Health, Belgrade, Yugoslavia.

Plasmid DNA analysis. At least one *Salmonella* strain of each serotype submitted from each country was arbitrarily chosen, and its plasmid DNA was isolated by the alkaline denaturation methods described previously (11, 13). The isolated DNA was analyzed on 0.8% agarose gels, stained with ethidium bromide, and photographed as described earlier (11).

To compare the plasmid content of strains analyzed on different agarose gels, we took the following approach. The alkaline denaturation methods that we used for DNA isolations always yielded a clearly distinguishable band of linear DNA fragments (chromosomal DNA). This allowed us to calculate the relative mobility of each plasmid DNA band with respect to the chromosomal band, which had by definition a mobility of 1.

The molecular sizes of the various plasmids were calculated from parabolic standard curves constructed by a computer program developed by Duggleby et al. (7). The molecular size standards were R27 (112 Md), R1 (62 Md), RP4 (36 Md), and ColE1 (4.2 Md). Their molecular sizes were taken from reference 6.

The DNA used for restriction enzyme analysis was isolated by a method modified from that of Portnoy et al. (21). All steps were performed in 10-fold volumes, and lysis was at 56°C for 20 min.

After polyethylene glycol precipitation, the pellet was dried and suspended in 8 ml of TE (50 mM Tris, 10 mM EDTA [pH 8.0]). Subsequently, 8 g of CsCl (Merck) and 1 ml of an ethidium bromide solution (5 mg/ml) was added. CsCl buoyant density gradients were formed at 40,000 rpm for 48 h at 15°C in a Beckman 50 Ti rotor. The high-density plasmid DNA band was visualized with a UV lamp (UVL-21; Ultraviolet Products, San Gabriel, Calif.) at 366 nm and removed by puncturing the gradient with a tuberculin syringe (Plastipak; Dublin, Ireland). The ethidium bromide was removed by four isopropanol extractions, and the plasmid DNA obtained by this method was dialyzed three times against 2 liters of 10 mM Tris–1 mM EDTA (pH 7.2) and stored at −30°C.

Restriction enzyme analysis. In general, 20 to 50 μl of the plasmid DNA preparations described above were digested with *Hin*dIII (Bethesda Research Laboratories, Gaithersburg, Md.) as recommended by the supplier. Restriction enzyme fragments were analyzed in 0.8 and 1% agarose gels (Bio-Rad Laboratories, Richmond, Calif.) for 1.5 h at 100 V in electrophoresis buffer (0.09 M boric acid, 0.09 M Tris, 0.025 M EDTA [pH 8.2]). Photographs were taken after staining with ethidium bromide. λ *Hin*dIII fragments served as molecular weight standards (7).

Isolation of outer membrane proteins. The outer membrane proteins were isolated as described by Achtman et al. (1). Basically, the cells were harvested and broken by sonication, and the outer membrane proteins were separated from other cellular proteins by Sarkosyl extraction and centrifugation. Subsequently, they were analyzed in a 10% acrylamide–0.26% methylene bisacrylamide–4 M urea-gel as described by Achtman et al. (1). The staining and destaining conditions and the sample buffer have also been previously described (1).

Infection of mice and LD_{50} determinations. All strains used for LD_{50} determinations were grown with aeration overnight at 37°C in L-broth. For oral infections, the cultures were washed once or concentrated 10-fold by centrifugation and suspension in 0.85% NaCl which also contained 3% sodium bicarbonate. Appropriate dilutions were also performed in this medium.

For oral infections, a 50-μl automatic pipette with a Tipac C20/TJ tip (Gilson, Villiers, France) was used to deposit the bacteria behind the upper incisors of BALB/c mice. For intraperitoneal infections, the bacteria were suspended in phosphate-buffered saline.

The mice used for infection studies were 3 to 4 weeks old and were obtained from the Robert von Ostertag Institut, Berlin, FRG. They were housed in plastic cages in groups of 10 and were fed Altrumin Mousefeed and water ad libitum.

The mice were observed for 28 days, and the LD_{50} values were calculated by the method of Reed and Munch (22).

Invasion tests. Invasion tests were performed on NMRI-Han mice that each received an oral dose of 10^8 bacteria. Three mice per day were sacrified at days 2, 4, 7, and 20 or when moribund, and the livers were removed aseptically. The organ was homogenized in 0.85% NaCl at a concentration of 50% (wt/vol) by using an Ultra Turrax (Janke and Kunkel, Staufen, FRG). Subsequently, the samples were

plated on Gassner plates containing 25 μg of nalidixic acid per ml.

Sereny test. The Sereny test (27) is frequently used as an assay for the invasive properties of *Shigella* (14) and *Yersinia* (15) species. Of an aerated overnight culture grown in L-broth, 50 μl was inoculated into the conjunctival sac of one eye each of two guinea pigs. A *Shigella flexneri* strain served as a positive control. Strains which elicited keratoconjunctivitis within 5 days were considered invasive. The diagnosis keratoconjunctivitis was given on the basis of an inflammation of the conjunctiva and an opaque cornea with or without purulent exudates. No special pathological examinations were performed.

Bactericidal assay. The fresh blood of three guinea pigs was obtained by heart puncture, allowed to clot for 1 h at room temperature followed by 3 h at 4°C, and then subjected to low-speed centrifugation. The serum was immediately used for assays of its bactericidal activity.

Bacteria were grown in L-broth at 37°C to 50 Klett units (5 × 10^8 CFU) and diluted 1,000-fold in phosphate-buffered saline. Of this dilution, 10 μl was added to 90 μl of serum and incubated at 37°C. At the times indicated below, 10 μl was plated for viable-cell count determinations. Complement-inactivated serum (heated for 10 min at 56°C) was always included as a control.

RESULTS

Plasmids in various serotypes. Although the genus *Salmonella* comprises more than 2,000 serotypes, only a few are encountered frequently, and these few serotypes cover more than 70% of all isolates (23; Kelterborn, abstr.). Consequently, we concentrated our investigations on the six most common serotypes. We also included *S. choleraesuis* because this serotype is well known to be highly pathogenic for humans (3).

From 33 laboratories in 29 countries distributed over five continents, 638 strains were received. The geographic distribution of these strains is shown in Table 1. The wide geographic distribution assured us that the strains did not belong to one epidemiological event. The source of the isolations was not specified, because studies on German isolates indicated that the plasmid DNA content of these serotypes is independent whether they originate from humans or animals (R. Helmuth, unpublished data). This has also been described by O'Brian et al. (19).

Representative gels obtained for *S. typhimurium*, *S. enteritidis*, *S. dublin*, and *S. choleraesuis* plasmid DNA preparations are shown in Fig. 1. Based on such gels, the relative mobilities of all plasmid species observed could be determined, and thus the percentages of strains carrying a plasmid with a given mobility (see above) could be calculated. The distribution of sizes obtained for every serotype is shown in Fig. 2.

S. typhimurium. A total of 60 antibiotic-sensitive *S. typhimurium* isolates from 25 countries were investigated, and 88% carried a plasmid which comigrated with plasmid R1 (molecular size, 62 Md). This plasmid had a relative mobility of 0.5 and was named pRQ28. A gel containing the plasmid DNA of pRQ28 is shown in Fig. 3. The plasmid also comigrates with the LT2 cryptic plasmid (28). Only one (1.6%) of all *S. typhimurium* strains carried no autonomous plasmid at all.

S. enteritidis. Of all isolates, 87% carried a plasmid with a relative mobility of 0.6, whereas 6% of all strains contained no plasmid (Fig. 2). The molecular mass of the plasmid was determined as 37 Md, and it was named pRQ29.

TABLE 1. Geographic distribution of *Salmonella* strains used for plasmid DNA and outer membrane protein preparations

Serotype	No. of isolates (no. of countries of origin/no. of continents of origin) analyzed for:	
	Plasmids	Outer membrane proteins
S. typhimurium	60 (25/5)	28 (16/4)
S. enteritidis	52 (23/5)	33 (17/4)
S. dublin	56 (22/5)	40 (20/5)
S. choleraesuis	34 (13/5)	39 (12/4)
S. infantis	40 (22/5)	30 (17/4)
S. panama	48 (19/4)	20 (17/4)
S. heidelberg	47 (20/5)	26 (9/4)

S. dublin. Of the strains, 89% carried a 56-Md plasmid as previously described (29). We named the plasmid pRQ30 (Fig. 3). Only five strains (9%) carried no plasmid at all.

S. choleraesuis. *S. choleraesuis* is rare in many countries, almost absent in Africa (Kelterborn, abstr.), and mainly isolated in Europe. Infections in humans are very severe, and generally a systemic infection occurs (3). We detected a 30-Md plasmid in every strain. The relative mobility was calculated as 0.7, and the plasmid was called pRQ20 (Fig. 3). No *S. choleraesuis* strain lacking that plasmid could be detected.

S. infantis. The majority (88%) of all 40 analyzed *S. infantis* strains carried no plasmid (Fig. 2). Nevertheless, we took one plasmid-carrying strain as a representative. It carried a 62-Md plasmid named pRQ31 (Fig. 3). Although only 4% of all *S. infantis* isolates carry this plasmid, it was the predominating one and was subsequently selected for further studies.

S. panama* and *S. heidelberg. The size distribution of the *S. panama* and *S. heidelberg* plasmids was different from the distributions of the serotypes described above. Here we could not demonstrate the existence of a uniform group of strains. In *S. panama*, plasmid-free strains were most frequently encountered (58% of all strains). A 21-Md plasmid called pRQ32 (Fig. 3) was present in 22% of all strains. In addition, these strains always carried a 3-Md cryptic plasmid called pRQ34.

Of all *S. heidelberg* isolates, 38% carried a 62-Md plasmid named pRQ33 (Fig. 3). It was the most frequently encountered plasmid in this serotype.

From these data, it was concluded that bacteria belonging to four of the seven serotypes investigated carried serotype-specific plasmids of a distinct molecular weight. These serotypes were *S. typhimurium*, *S. enteritidis*, *S. dublin*, and *S. choleraesuis*. Most *S. infantis* isolates were primarily plasmid free, whereas *S. panama* and *S. heidelberg* isolates showed plasmid DNAs with heterogeneous sizes.

Plasmid DNAs from one representative of each serotype are compared in Fig. 3. For those serotypes with a common plasmid, at least five strains were chosen arbitrarily, and their plasmids were tested after *Hin*dIII digestion. In all cases, the plasmids yielded the same restriction fragment patterns within one serotype. An example of this type of analysis for seven *S. dublin* isolates is given in Fig. 4.

Outer membrane protein profiles. Outer membrane protein profiles show diversity among *E. coli*, *H. influenza* and *Vibrio cholerae* isolates (1, 2, 16). The outer membrane proteins of *Salmonella* isolates were therefore analyzed (Table 1). For *S. typhimurium*, *S. choleraesuis*, *S. infantis*, *S. panama*, and *S. heidelberg*, it turned out that within the

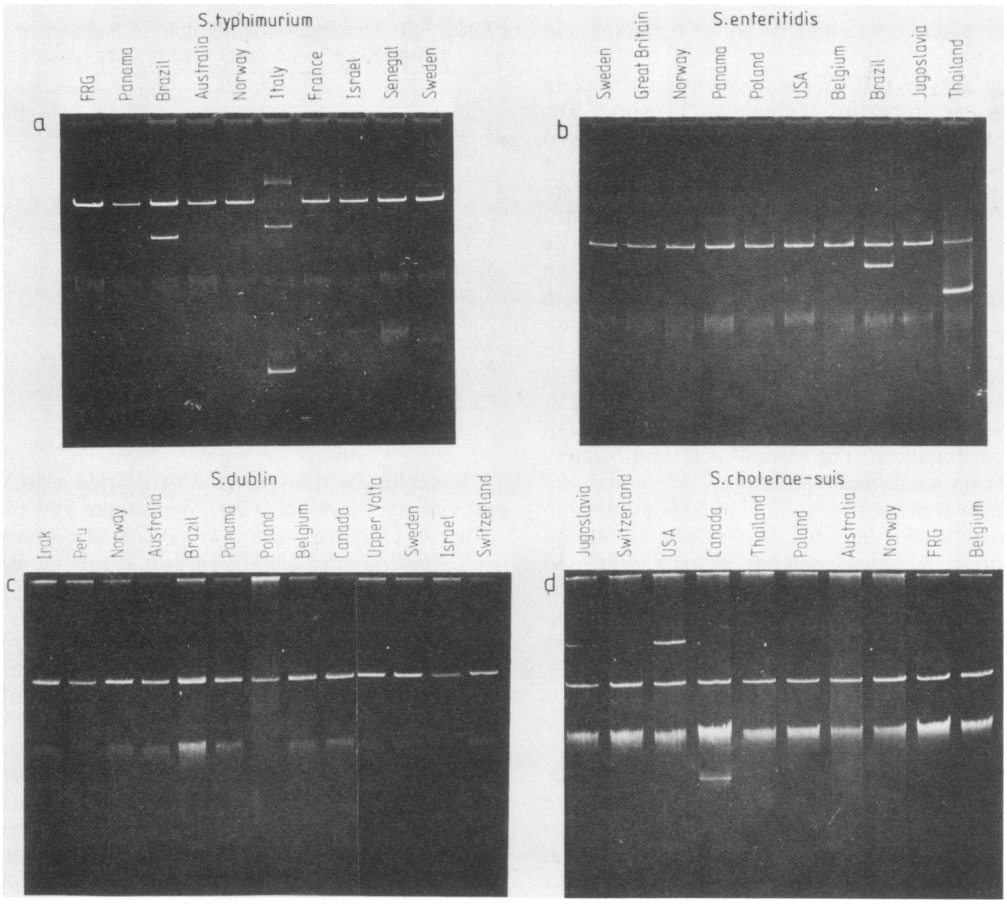

FIG. 1. Typical plasmid DNA gels showing plasmid patterns in four *Salmonella* serotypes.

serotypes the major outer membrane protein pattern was uniform for all strains tested. The outer membrane protein profiles of representative strains are shown in Fig. 5. Two outer membrane protein profiles could be detected in each of *S. enteritidis* and *S. dublin*. The pattern characteristic for 30 plasmid-containing strains and 1 plasmid-free strain of *S. enteritidis* are shown in track 2 of Fig. 5. Two plasmid-free strains, however, showed the membrane pattern given in track 3 of Fig. 5. In these latter strains, the 41.7-kilodalton (kd) band was substituted for by a band which migrated slightly slower than the 40-kd protein. For *S. dublin*, it was shown that the 35 plasmid-containing strains and 1 plasmid-free strain showed the pattern given in Fig. 5, track 4, whereas 4 other plasmid-free strains lacked the 38.7- and 41-kd proteins and carried an additional 40-kd band (Fig. 5, track 5). The comparison of membrane patterns between the seven serotypes also revealed very striking similarities. All *S. typhimurium* (track 1), *S. panama* (track 8), and *S. heidelberg* (track 9) isolates showed the same profile. This profile was also identical with the one shown by two plasmid-free *S. enteritidis* (track 3) and four plasmid-free *S. dublin* strains (track 5). A second pattern was shown by the majority of *S. enteritidis* (track 2) and all *S. choleraesuis* strains (track 6). All *S. infantis* strains defined the third type (track 7), and all plasmid-carrying strains and one plasmid-free *S. dublin* strain defined the fourth type of pattern (track 4). In general the outer membrane profiles were quite similar among the serotypes and would not be very useful for epidemiological studies. This contrasts with the results found for other bacterial species (1, 2, 5).

The 37-kd band was heat modifiable and reacted with monoclonal antibodies directed against the *E. coli* outer membrane protein OmpA (A. Moll, unpublished data).

Correlation between plasmid content and pathogenicity. To test whether the serotype-specific plasmids found in *S. typhimurium*, *S. enteritidis*, *S. dublin*, and *S. choleraesuis* and the predominant plasmids in *S. infantis*, *S. panama*, and *S. heidelberg* are associated with pathogenicity, mouse infection studies, invasion tests, and serum resistance experiments were performed. One arbitrarily chosen representative plasmid-carrier strain and a plasmid-free strain from each serotype were tested in detail. For a more qualitative characterization of virulence properties, two other plasmid carriers and (when possible) two other plasmid-free strains were selected. This characterization included a confirmation of their virulence for mice, their invasive properties, and serum resistance. The data obtained for the properties of the intensively investigated representative strains are given in Table 2. These properties were always confirmed by the data obtained for the additional strains studied only qualitatively.

From the LD_{50} data shown, one could conclude that members of the seven serotypes can be divided into virulent and avirulent strains. After oral infection, virulent strains have LD_{50} values of less than 2.0×10^4 CFU. Such highly virulent strains are only found among strains carrying the serotype-specific plasmids described above. Plasmid-free strains of *S. typhimurium*, *S. enteritidis*, and *S. dublin* resemble strains of *S. infantis*, *S. panama*, and *S. heidelberg* in being avirulent for BALB/c mice after oral infection (LD_{50} values of more than 5×10^8 CFU).

The same behavior was found with the intraperitoneal-infection model of NMRI-Han mice except that the plasmid-carrying and plasmid-free *S. typhimurium* strains did not differ significantly.

A similar relationship between the virulence properties and the plasmid content could also be shown for the invasive properties of the strains. For *S. typhimurium*, *S. enteritidis*, and *S. dublin*, only the plasmid carriers were detectable in the livers of animals infected per os, and only they gave positive responses in the Sereny test. The overall pathological lesions observed for the plasmid-carrying *S. enteritidis* isolates resembled those of the included *Shigella* control strains. Such responses were also shown by *S. choleraesuis* isolates, but the lack of a plasmid-free strain prevented our drawing any conclusions about the serotype-specific plasmid pRQ20 and about the virulence of the strains. *S. heidelberg* was the only serotype in which a mild inflammation of the eye in the Sereny test could be detected irrespective of plasmid DNA content. For *S. heidelberg*, however, no invasion of the liver could be demonstrated. *S. infantis* and *S. panama* were shown to be noninvasive by both assays.

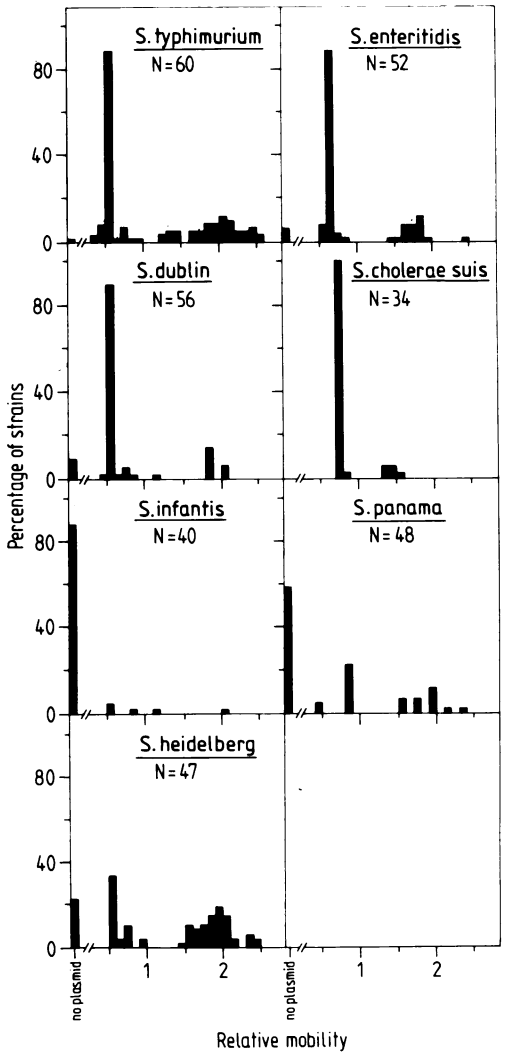

FIG. 2. Percent distributions of relative mobilities of plasmids detected in seven *Salmonella* serotypes. N, Number of strains investigated.

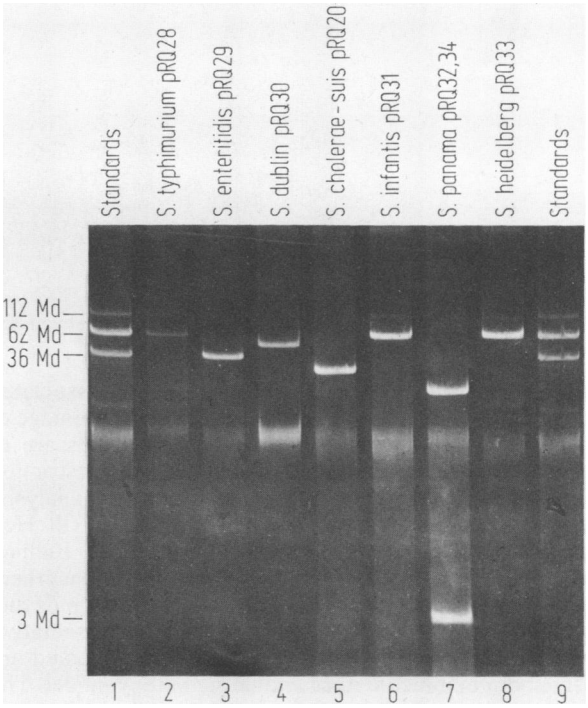

FIG. 3. Comparison of serotype-specific plasmids of seven selected representative strains.

The involvement of the serotype-specific plasmids was also obvious in serum resistance experiments. Only the strains of *S. enteritidis*, *S. dublin*, and *S. choleraesuis* which carried plasmids were absolutely unaffected by the bactericidal activity of 90% guinea pig serum. *S. typhimurium* strains carrying pRQ28 and *S. heidelberg* strains carrying pRQ33 were also more serum resistant than the plasmid-free strains but showed no growth in the serum. All other strains except *S. infantis* 515/82 exhibited a more or less pronounced serum sensitivity. Strain 515/82 was the only *S. infantis* strain among two other plasmid-free strains that was slightly serum resistant, and it might well carry serum resistance genes on its chromosome.

DISCUSSION

It has been shown that four out of seven important *Salmonella* serotypes, namely *S. typhimurium*, *S. dublin*, *S.*

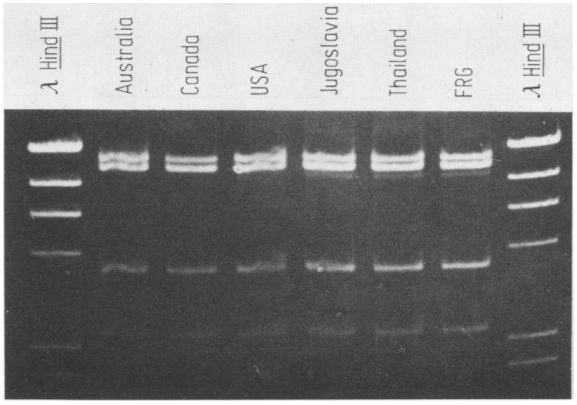

FIG. 4. *Hin*dIII restriction enzyme patterns of eight arbitrarily chosen *S. dublin* isolates. The bright DNA band shown by the strain from the United States represents an uncut, small, cryptic plasmid.

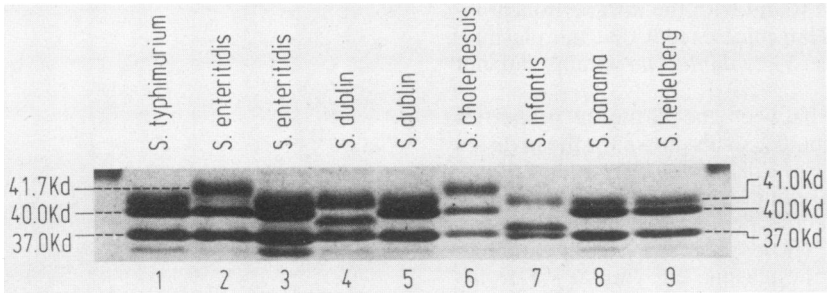

FIG. 5. Outer membrane protein profiles detected in seven *Salmonella* serotypes. The low-molecular-weight band in lane 3 was never detected in other preparations of the same strain.

enteritidis, and *S. choleraesuis*, carry virulence-associated plasmids. These plasmids are present in a high percentage of strains all over the world. The virulence plasmids are of different sizes, ranging from 30 to 62 Md. The restriction enzyme and electron-microscopic heteroduplex analysis, however, shows large regions of DNA homology (R. Helmuth, (R. G. Morelli, M. A. Montenegro, and E. Bulling, manuscript in preparation). It has also been shown that there is a correlation between the presence of these plasmids and virulence, because all arbitrarily chosen representative strains carrying the plasmids are virulent for mice and are invasive both in infected mice and in the Sereny model. The plasmid carriers are also serum resistant. All plasmid-free isolates of each serotype, in contrast, are less virulent, are not invasive, and are serum sensitive. From these data it can be concluded that in the four serotypes mentioned above, natural *Salmonella* isolates exist as high-virulence or low-virulence subclones. The low-virulence strains might be derived from the high-virulence ones by the loss of the serotype-specific plasmid which carries virulence determinants. We do not know whether this loss happens in nature or is the result of the history of the strains in the laboratory. Based on our observations, it seems very likely that this loss happens in nature, because our attempts to cure plasmid-carrying strains by various methods had been, until now, unsuccessful. Even transposon-tagged plasmids turned out to carry their drug resistance genes very stably. To show conclusively that the plasmids observed are involved in the

TABLE 2. Virulence properties of plasmid-carrying and plasmid-free *Salmonella* isolates

Serotype and strain	Plasmid[a]	LD_{50}		Maximum no. of bacteria per gram of liver[d]	Sereny test[e]	% Survival in 90% serum[f]
		Oral[b]	Intraperitoneal[c]			
S. typhimurium						
353/82	pRQ28	1.4×10^3	1.2×10^4	1.6×10^3	++	66
955/81	—	7.0×10^8	7.8×10^4	<20	+	<1
S. enteritidis						
4724	pRQ29	2.2×10^3	3.6×10^2	1.0×10^4	+++	280
2930	—	$>3.4 \times 10^9$	4.0×10^5	<20	−	<1
S. dublin						
643	pRQ30	1.2×10^3	4.0×10^1	8.0×10^4	+	432
2526/25	—	$>3.5 \times 10^9$	2.5×10^6	<20	−	14
S. choleraesuis						
807	pRQ20	1.4×10^4	2.0×10^2	1.0×10^6	+++	375
S. infantis						
2240/82	pRQ31	$>4.1 \times 10^9$	9.0×10^7	<20	−	4
515/82	—	3.8×10^9	9.0×10^7	<20	−	120
S. panama						
672/82	pRQ32	$>1.6 \times 10^9$	1.4×10^6	<20	−	25
321/82	—	$>2.5 \times 10^9$	4.0×10^6	<20	−	22
S. heidelberg						
278/82	pRQ33	$>3.2 \times 10^9$	4.4×10^4	<20[g]	+	66
105/82	—	$>2.3 \times 10^9$	5.6×10^5	<20[h]	+	3

[a] —, Plasmid-free strain.
[b] BALB/c mice.
[c] NMRI-Han mice.
[d] Detected at day 2, 4, 7, or 20.
[e] −, No reaction; +, inflammation; ++, keratoconjunctivitis; +++, purulent keratoconjunctivitis.
[f] After 3 h at 37°C. Each number given represents the median of at least two experiments.
[g] In one of six experiments, 320 bacteria per g of liver was detected.
[h] In one of six experiments, 110 bacteria per g of liver was detected.

pathogenicity of salmonellosis would require such cured strains and the reintroduction of the plasmids into them. Such experiments and a more detailed description of the virulence factors are the aims of our continued research. The paper presented here concentrates, however, on the virulence properties of natural isolates and establishes their plasmid epidemiology.

In addition to the increased virulence provoked by these plasmids, one can speculate that they are useful natural vehicles for transposons carrying drug resistance genes. We have some indications that many R-factors in *Salmonella* species are derived from the serotype-specific plasmids described.

We could detect no predominating plasmid in *S. infantis*. Of the strains, 88% were plasmid free. *S. infantis*, however, does not invade the organs of the infected mice and thus does not need plasmids carrying genes for invasion or serum resistance to exhibit its pathogenic mechanisms.

S. panama and *S. heidelberg* also showed no virulence plasmids. Furthermore, it was observed that all three arbitrarily tested plasmid-carrying and plasmid-free strains were noninvasive. In *S. heidelberg* an increased serum resistance could be detected, and the intraperitoneal LD_{50} varied only 10-fold in relation to the 51-Md plasmid pRQ33, so it seems that the behavior of this serotype is similar to that shown by *S. typhimurium*. *S. heidelberg* isolates are, however, avirulent and noninvasive after oral infection of mice.

The serotype-specific plasmids described can be very useful for epidemiological purposes. Frequently observed D1-O-forms, for example, can be differentiated by an analysis of their plasmid DNA content. This enables the differentiation of *S. enteritidis*, *S. dublin*, or any other serotype.

The major outer membrane protein profiles are, within a given serotype, very homogeneous and thus are not useful epidemiological markers. This observation differs from the ones made for other bacterial genera, but might be explained by the fact that the *Salmonella* serology is very well developed. In other systems, unknown O antigens might exist, and, consequently, a serologically homogeneous group of bacteria might turn out to be inhomogeneous by other criteria.

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LITERATURE CITED

1. **Achtman, M., A. Mercer, B. Kusecek, A. Pohl, M. Heuzenroeder, W. Aaronson, A. Sutton, and R. P. Silver.** 1983. Six widespread bacterial clones among *Escherichia coli* K1 isolates. Infect. Immun. **39**:315–335.
2. **Barenkamp, S. J., R. S. Munson, and D. M. Granoff.** 1981. Subtyping isolates of *Haemophilus influenzae* typed by outer membrane protein profiles. J. Infect. Dis. **143**:668–676.
3. **Bernard, D. D., R. Dulbecco, H. N. Eisen, and H. S. Ginsberg.** 1980. *In* B. D. Davis (ed.), Microbiology, 3rd ed., J. D. Lippincott Co., Philadelphia.
4. **Broda, P.** 1979. Plasmids. W. H. Freeman and Co., Oxford, N.Y.
5. **Buchanan, T. M., and J. F. Hildebrandt.** 1981. Antigen-specific serotyping of *Neisseria gonorrhoeae*: characterization based upon principal outer membrane protein. Infect. Immun. **32**: 985–994.
6. **Bukhari, A. I., J. A. Shapiro, and S. L. Adhya (ed.).** 1977. DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Duggleby, R. G., H. Kinns, and J. S. Rood.** 1981. A computer program for determining the size of DNA restriction fragments. Anal. Biochem. **110**:49–55.
8. **Ewing, W. H., and W. J. Martin.** 1974. *Enterobacteriaceae*, p. 189–221. *In* E. H. Lenette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
9. **Gassner, G.** 1918. Ein neuer Dreifarbnährboden zur Typhus-Ruhr Diagnose. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **80**:219–222.
10. **Hale, T. L., P. J. Sansonetti, P. A. Schad, S. Austin, and S. B. Formal.** 1983. Characterization of virulence plasmids and plasmid-associated outer membrane proteins in *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. Infect. Immun. **40**:340–350.
11. **Helmuth, R., R. Stephan, E. Bulling, W. J. van Leeuwen, J. D. A. van Embden, P. A. M. Guinée, D. Portnoy, and S. Falkow.** 1981. R-factor cointegrate formation in *Salmonella typhimurium* bacteriophage type 201 strains. J. Bacteriol. **146**:444–452.
12. **Jones, G. W., D. K. Rabert, D. M. Svinarich, and H. J. Whitfield.** 1982. Association of adhesive, invasive, and virulent phenotypes of *Salmonella typhimurium* with autonomous 60-megadalton plasmids. Infect. Immun. **38**:476–486.
13. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
14. **Machel, D. C., F. L. Lorreine, and L. A. Venice.** 1961. The use of the guinea pig conjunctivae as an experimental model for the study of virulence of *Shigella* organisms. Am. J. Hyg. **73**: 219–223.
15. **Mäki, M., T. Vesikari, J. Rantala, C. Sundqvist, and P. Grönroos.** 1983. Pathogenicity of 42-44 Md plasmid positive and negative *Yersinia pseudotuberculosis* I and *Yersinia enterocolitica* 0:8 and 0:9 studied in the guinea pig eye model (Sereny test). Acta Pathol. Microbiol. Immunol. Scand. Sect. B. **91**:241–244.
16. **Manning, P. A., F. Imbesi, and D. Haynes.** 1982. Cell envelope proteins in *Vibrio cholerae*. FEMS Microbiol. Lett. **14**:159–166.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Munford, R. S., C. M. Patton, and G. W. Gorman.** 1975. Epidemiological studies of serotype antigens common to group B and C *Neisseria meningitidis*. J. Infect. Dis. **131**:286–290.
19. **O'Brian, T. F., J. D. Hopkins, E. S. Gilleece, A. A. Medeiros, R. L. Kent, B. O. Blackburn, M. B. Holmes, J. P. Reardon, J. M. Vergerant, W. L. Schell, E. Christenson, M. L. Bisset, and E. V. Morse.** 1982. Molecular epidemiology of antibiotic resistance in *Salmonella* from animals and human beings in the United States. N. Engl. J. Med. **307**:1–6.
20. **Portnoy, D. A., H. F. Bland, D. T. Kingsbury, and S. Falkow.** 1983. Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. J. Infect. Dis. **148**:297–304.
21. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**: 775–782.
22. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
23. **Rowe, B.** 1982. *Salmonella* surveillance. W.H.O. programme. Central Public Health Laboratory, London.
24. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1981. *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. Infect. Immun. **34**:75–83.
25. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
26. **Seiler, A., and R. Helmuth.** 1984. Development and localization of multiresistance in *Salmonella dublin*, p. 309–313. *In* Proceedings of the CEC Scientific Workshop on Priority Aspects of Salmonellosis Research, October 1983. Commission of the European Communities, Brussels, Belgium.
27. **Serény, B.** 1955. Experimental *Shigella* keratoconjunctivitis. A preliminary report. Acta Microbiol. Acad. Sci. Hung. **2**:293–296.
28. **Spratt, B. G., and R. J. Rowbury.** 1973. The plasmid of

Salmonella typhimurium LT2. Mol. Gen. Genet. **121**:347–353.
29. **Terakado, N., T. Sekizaki, K. Hashimoto, and S. Naitoh.** 1983. Correlation between the presence of a fifty-megadalton plasmid in *Salmonella dublin* and virulence for mice. Infect. Immun. **41**:443–444.
30. **van Embden, J. D. A., W. J. van Leeuwen, and P. A. M. Guinée.** 1976. Interference with propagation of typing bacteriophages by extrachromosomal elements in *Salmonella typhimurium* bacteriophage type 505. J. Bacteriol. **127**:1414–1426.
31. **Yancey, R. J., S. A. L. Breeding, and C. E. Lankford.** 1979. Enterochelin (enterobactin): virulence factor for *Salmonella typhimurium*. Infect. Immun. **24**:174–180.